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STUDIES OF THE OUTER MEMBRANE PROTEINS OF CAMPYLOBACTER JEJUNI
FOR VACCINE DEVELOPMENT

MIDTERM REPORT

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FOREWORD

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.



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A. Progress

1. Studies of erythromycin-resistance in *Campylobacter*s.

In collaboration with colleagues at the University of Washington in Seattle, and AFRIMS in Bangkok, we participated in studies to define frequency and mechanism of erythromycin-resistance in *Campylobacter* isolates. Forty-two erythromycin resistant (EmR) strains of *Campylobacter* from domestic- and foreign-acquired human infections, AIDS patients and poultry were examined. Two naturally occurring resistance patterns were found: (1) high level resistance to erythromycin, tylosin, clindamycin and vernamycin B (N=41) and (2) resistance to erythromycin and vernamycin B, but not tylosin or clindamycin (N=1). The predominant phenotypic pattern suggested the presence of an rRNA methylase; however, DNA probes to common bacterial genes encoding erythromycin resistance did not hybridize with the EmR *Campylobacter*, suggesting a novel resistance determinant. Erythromycin-resistant isolates were selected by serial passage on erythromycin containing media from two erythromycin-sensitive strains (related by plasmid profiles and serotype to naturally occurring EmR strains); however, the pattern and magnitude of resistance in the *in vitro* isolates was different from the *in vivo* resistant isolates. Examination of 660 isolates collected in King County, Washington, revealed that EmR *C. jejuni* were not a substantial problem in human or poultry isolates (0% and 2%) but that EmR *C. coli* represented 12% and 30% of human and poultry isolates, respectively.

C. coli is a much more significant problem in developing countries, where U.S. troops may be exposed. Previous studies from AFRIMS in Bangkok indicate that the *C. coli* strains isolated from Thailand are often erythromycin-resistant. High frequency resistance and emergence of resistance to new agents supports the concept that vaccination of troops against campylobacters will be an important preventive measure.

2. The role of Cholera-like Enterotoxin in *Campylobacter* Enteritis in Thailand.

Although *Campylobacter* enteritis is usually an inflammatory process in developed countries, watery diarrhea is common in the developing world. Some investigators have found strains of *Campylobacter* that produce a cholera-like toxin which may be responsible for watery diarrhea. We investigated by ELISA the immune response to cholera toxin and to surface exposed antigens of *C. jejuni* in children with acute diarrhea in Bangkok. Sera from children infected with *Campylobacter* (n=29, mean age 13.5 mo) and infected with *Shigella* (n=26, mean age 23.1 mo) were collected during the acute phase, and 2 and 4 weeks after the diarrheal episode. By

ELISA, children infected with *Campylobacter* but not *Shigella* showed a significant increase of antibody levels to *C. jejuni* specific antigens in all three immunoglobulin classes ($p < 0.01$). In the *Campylobacter* infected group antibody levels were similar whether watery or bloody diarrhea was present, however, children with diarrhea for more than 72h or older than two years of age were less reactive to *C. jejuni* antigens. Neither *Campylobacter*- nor *Shigella*-infected children showed any increase in antibody levels to cholera toxin. Furthermore, 29 *Campylobacter* isolates from this study did not hybridize with LT or Shiga-like I or II probes. In total, these data argue against a role for enterotoxin in the pathogenesis of *Campylobacter* enteritis in developing countries. This represents our third study that has indicated that enterotoxins are not important in the pathogenesis of *Campylobacter* diarrhea. Taken together with negative studies by other investigators, this study indicates that it is no longer worthwhile to pursue enterotoxin as a vaccine component.

3. Investigation of an outbreak of *Campylobacter jejuni* in Multiple Oklahoma Prisons.

An outbreak of 104 cases of *Campylobacter jejuni* gastroenteritis occurred in 1988 in four Oklahoma prisons. Cases were defined as persons with diarrhea and three or more other symptoms. One hundred inmates (97%) had diarrhea, 69 (67%) had fever, and 24/114 (20%) had positive stool cultures. Case-control studies showed that consumption of pasteurized milk from the prison dairy farm was associated with illness; 90 (87%) cases consumed milk, while only 64 (60%) controls consumed milk (Odds ratio OR = 2.4, $p < .05$). Manure samples of cattle from the prison dairy farm grew *C. jejuni* strains antigenically similar (Penner 8, 1W; Lior 2) to the human isolates. We performed serologic testing of samples provided from culture-positive, culture-negative (clinical) cases and from unaffected controls using an ELISA based on *C. jejuni* antigens. The culture-positive and clinical cases were more likely to have positive serologic ELISA tests for *C. jejuni* specific immunoglobulin ($p < .0001$ for IgA, $p < .01$ for IgG, and $p < .01$ for IgM) than the controls. Although a deficit in the pasteurization process was not identified, the milk had elevated coliform counts from samples taken on the day the implicated milk was distributed to the prisons. The results of these findings, implicating pasteurized milk as a vehicle of spread, underscore the importance of proper pasteurization techniques and other proper food handling procedures. This outbreak investigation also points to the utility of serologic tests for diagnosing *Campylobacter* infection, which could be useful in field settings.

4. Identification, purification, and characterization of major antigenic proteins of *Campylobacter jejuni*.

Our studies of the surface proteins of *C. fetus* and *H. pylori* have indicated that a group of proteins external to the outer membrane are important antigens and provide significant functions for these bacteria. From our previous work, we have identified 4 proteins in the acid-extract from *C. jejuni* which may be vaccine candidates. These are PEB1 (migrates at 28 kDa), PEB2 (29kDa) PEB3 (30 kDa), PEB4 (31kDa). In this work, we have developed techniques for isolation of these proteins, and have found that two of the surface proteins may be valuable vaccine candidates.

This work was presented at the NIH Symposium on *Campylobacter jejuni* at Asilomar, California and has been published in the *Journal of Biological Chemistry* (reprint attached).

The major points of the publication are that:

1. We have purified 4 different surface proteins migrating at 28 (PEB1), 29 (PEB2), 30 (PEB3), and (PEB4) kilodaltons.
2. All are highly basic proteins.
3. Most *C. jejuni*-infected persons but not controls sero-converted to PEB1 and PEB3.
4. Antiserum to PEB1 recognized all *C. jejuni* and *C. coli* strains but no other *Campylobacter* strains.

Therefore, the *C. jejuni* PEB1 and possibly PEB3 proteins appear to be candidate antigens for both a *Campylobacter* vaccine and for serologic assays for the pathogen.

5. Purification of a PEB1 analog in *C. coli*.

As reported above, we have purified a 28 kDa protein from *C. jejuni* that is commonly recognized by convalescent sera from *C. jejuni*- and *C. coli*-infected patients, and conserved in both species. We now purified a 28 kDa molecule from *C. coli* using cationic exchange FPLC. This molecule shares characteristics with the 28 kDa molecule of *C. jejuni* (PEB1-J) as follows: (i) Both are sensitive to proteinase K digestion, indicating that the major component is a protein. (ii) The two molecules have similar amino acid composition with basic amino acids predominating. (iii) The molecular weight of the *C. coli* protein is 28 kDa under non-denaturing conditions. The N-termini of the *C. coli* PEB1-C and *C. jejuni* proteins share 14 identical and 6 conserved amino acid residues in the first 20 residues, suggesting

that quantitative differences between *C. jejuni* and *C. coli* cells as determined by immunological assays may be due to antigenic differences. The N-terminus of the *C. coli* protein shared significant sequence homology with stress (heat-shock) proteins including *E. coli* *uvrA*, *E. coli* ATP-dependent protease La (*lon*) and *Bacillus megaterium* major heat shock protein (*dnaK*). These data suggest that the conserved low molecular weight proteins of *C. jejuni* and *C. coli* may represent stress proteins, explaining their antigenic characteristics.

6. Studies of serologic response to *Campylobacter jejuni* lipopolysaccharides.

We studied the serological response to purified lipopolysaccharide from four *Campylobacter* strains in 34 adults with inflammatory enteritis in Denver, using an enzyme-linked immunosorbent assay (ELISA) for detection of IgA, IgG, and IgM. LPS was prepared by the hot phenol-water method and each preparation had <3% protein contamination. Two LPS preparations were of the same *C. jejuni* O-group antigen (O:4 and O:5) as were isolated from seven patients each, one preparation was from a non-typable O-group (O:NT) strain, and the other from O:1. Comparing acute and convalescent sera for the 22 *Campylobacter*-infected patients, mean optical density levels rose significantly in all three immunoglobulin classes to all 4 LPS preparations. There was no significant rise in any immunoglobulin class to any LPS preparation in the patients infected with other pathogens. Seroconversion in one or more immunoglobulin class to any LPS was found in 17 of 22 *Campylobacter*-infected versus none of seven non-*Campylobacter*-infected patients ($p < .02$). Significant responses to O:4 and O:5 LPS were seen in the seven patients each infected with homologous O-group *Campylobacter* strains and to a variable degree in the 15 patients infected with *Campylobacter* heterologous O-groups. Our results indicate that intestinal infection with *Campylobacter* induces a serologic response to homologous and heterologous O-group LPS; the heterologous response may be due to shared *Campylobacter* core LPS antigens. The data, in conjunction with our earlier volunteer studies indicate that there are conserved epitopes in the *C. jejuni* LPS core that could have value for vaccine development.

7. Identification and purification of a heat shock protein from *Campylobacter jejuni*.

This work is a continuation of our efforts in screening surface proteins of *C. jejuni* for vaccine candidates. We purified a 36 kDa protein from the glycine extract of strain 81-176 (which is the same strain from which PEB1 to 4 were purified), by using preparative SDS-PAGE (Figure 1). The purified 36 kDa protein which we now call PEB5, was tested for its ability to induce antibody

response in humans during natural infection. By using Western blotting, 3 of 3 convalescent sera from *C. jejuni*-infected patients recognized this band. The amino terminus of this protein was sequenced up to 28 amino acid residues. Similar to PEB1 to 4, the mature 36 kDa protein did not start with methionine at its N-terminus, indicating that post-translational cleavage of a leader peptide probably occurs. The N-terminus of 36 kDa protein shared very significant homology with a number of heat shock proteins of the cpn60 chaperonin family (Figure 2), including GroEL protein of *E. coli* (75% identity), 57 kDa chlamydial hypersensitivity antigen (75%), 65 kDa antigen (cell wall protein A) of *Mycobacterium tuberculosis*, and *M. leprae* (66%) and mitochondrial protein p1 precursor of human and Chinese hamster cells (64%), and rubisco subunit binding-protein alpha subunit precursor of wheat (57%). The 36 kDa protein (PEB5) is a possible common antigen in *C. jejuni*, and by analogy to the other chaperonin proteins could play a role in the immunopathogenesis of infection.

8. Cloning PEB1 from *Campylobacter jejuni*.

As reported previously, we have identified and purified four *C. jejuni* proteins, PEB1 to 4. Two of these proteins, PEB1 and PEB3 were recognized by a majority (80%) of convalescent sera from *C. jejuni* or *C. coli*-infected patients and appear to have potential as vaccine candidates. In the last report we provided more evidence that PEB1 also was conserved in *C. coli* by purification and characterization of a PEB1-like protein from a *C. coli* strain. Based on this information, we have concentrated our efforts on cloning the gene for PEB1 in *E. coli* in order to develop a system for production of PEB1 in large quantities to meet the need for this antigen in vaccine trials in both animals and humans. To accomplish this goal, genomic DNA was prepared from *C. jejuni* strain 81-176, and sheared by sonication. Fragments ranging from 1 to 10 kb were isolated using a Sepharose CL2B column, ligated to *EcoRI* linkers and inserted into the *EcoRI* site in lambda gt11. Recombinant phages were packaged and used to transform *E. coli* Y1088. The resulting bank was amplified in *E. coli* Y1090 cells and screened with polyclonal antibody to PEB1 protein from a rabbit hyperimmunized with PEB1. Two positive recombinant plaques were selected, purified to clonality, and found to contain the same 2.6 kb insert from *C. jejuni* (Figure 3). The insert from clone 1 was subcloned into pUC19 to create pPB1. The resulting clones were mapped by a variety of restriction enzymes (Figure 4). After deletion mutations, plasmids containing 1.6 kb inserts in both orientations were identified and called pPB119 and pPB219. We next used Western blotting to confirm the production of PEB1 in pPB119 and pPB219, and to characterize the size of the gene product. Both pPB119 and pPB219 produced proteins of 28 kDa recognized by the antiserum specific for PEB1. The proteins were produced in the absence of any induction, suggesting that the insert contained the necessary promoter for transcription of the full length PEB1 gene. We then generated a series of deletion mutants from pPB119 by using

both restriction enzymes based on the physical map shown in Figure 2 and unidirectional deletion by exonuclease III to localize the PEB1 gene. We found that the 1.2 kb fragment between *EcoRI* and *NcoI* sites still produced the full length PEB1 protein of 28 kDa. Since there is a *lacZ* promoter located upstream of the insert, we asked whether induction of transcription from this promoter could improve production of PEB1 in these recombinant *E. coli*. In the absence of the inducer (IPTG) for the *lacZ* promoter, both pPB119 and pPB219 were able to produce PEB1, suggesting the insert may contain an appropriate promoter for PEB1 in addition to its structural gene. When induced by IPTG, production of PEB1 was significantly increased in pPB119 but not in pPB219, as judged by Western blotting. These data indicate that transcription of the PEB1 gene is in same orientation as *lacZ* in pPB119, but is in the opposite orientation in pPB219. Our future plans for this work are: (i) to improve the production of PEB1 (ii) develop a method for large scale purification of PEB1 protein from pPB119, and (iii) to sequence the PEB1 gene.

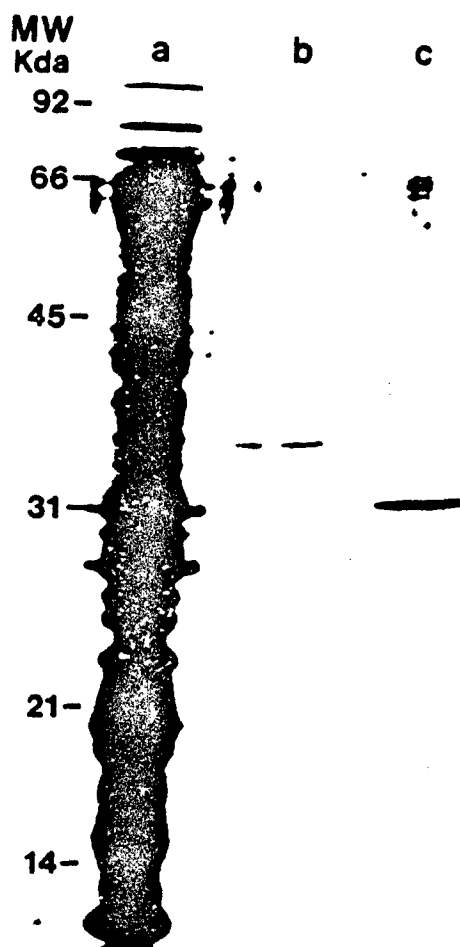


Figure 1

SDS-PAGE of PEB3 and PEB5 proteins purified from glycine extract of *C. jejuni* strain 81-176 using preparative SDS-PAGE. Lanes are: glycine extract (a); purified PEB5 (b); purified PEB3 (c); Protein bands were resolved by silver staining. Molecular weight markers are shown at left.

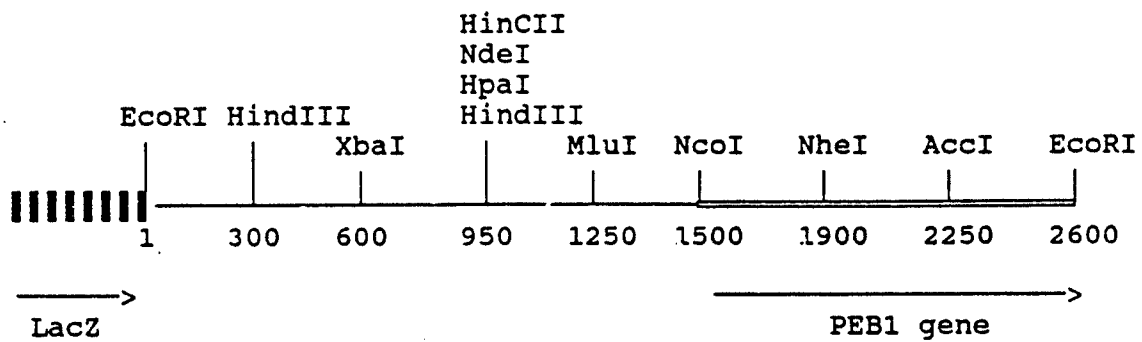


Figure 4. Physical map of *C. jejuni* insert in plasmid pPB1.

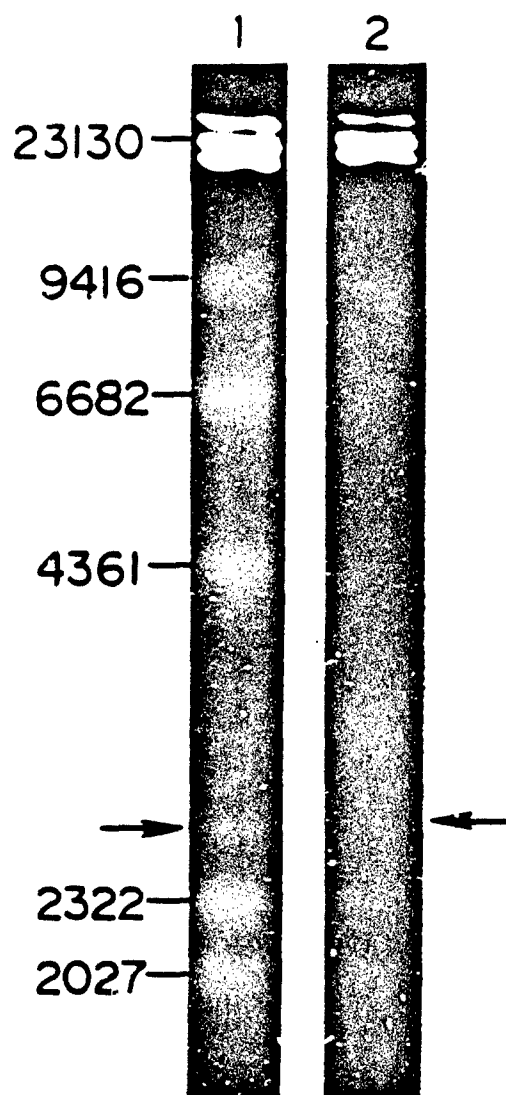


Figure 3 Agarose gel electrophoresis of phage DNA of two positive recombinants selected from lambda gt11 gene bank. Lanes show *EcoRI* digestions of phage DNA from clone 1 (lane 1) and clone 2 (lane 2). The *C. jejuni* DNA inserts were released from the recombinant phage DNA after *EcoRI* digestion (as shown by arrows) and migrated with about 2600 base pairs. DNA molecular weight markers in base pairs are shown at left.

		Identity with PEB5
PEB5 (36 KDa) of <u>C. jejuni</u>	(1) <u>A</u> <u>A</u> <u>N</u> <u>S</u> -- <u>D</u> <u>E</u> <u>K</u> <u>I</u> <u>G</u> <u>N</u> <u>L</u> <u>I</u> <u>A</u> <u>D</u> <u>A</u> <u>M</u> <u>E</u> <u>K</u> <u>V</u> <u>G</u> <u>K</u> <u>D</u> <u>G</u> <u>V</u> <u>I</u> <u>T</u> <u>V</u> <u>E</u> (28) ::: :: : ::: :: :::: :::::	
Groel protein of <u>E. coli</u>	(150) <u>S</u> <u>A</u> <u>N</u> <u>S</u> -- <u>D</u> <u>E</u> <u>T</u> <u>V</u> <u>G</u> <u>K</u> <u>L</u> <u>I</u> <u>A</u> <u>E</u> <u>A</u> <u>M</u> <u>O</u> <u>K</u> <u>V</u> <u>G</u> <u>K</u> <u>E</u> <u>G</u> <u>V</u> <u>I</u> <u>T</u> <u>V</u> <u>E</u> (177) 75% :: : ::::: ::::: : ::::	
57 KDa <u>Chlamydial</u> hypersensitivity antigen	(151) <u>S</u> <u>A</u> <u>N</u> <u>N</u> -- <u>D</u> <u>A</u> <u>E</u> <u>I</u> <u>G</u> <u>N</u> <u>L</u> <u>I</u> <u>A</u> <u>E</u> <u>A</u> <u>M</u> <u>E</u> <u>K</u> <u>V</u> <u>G</u> <u>K</u> <u>N</u> <u>G</u> <u>S</u> <u>I</u> <u>T</u> <u>V</u> <u>E</u> (178) 75% :: : : ::: ::: ::: :::::	
65 KDa cell wall pprotein A of <u>M. tuberculosis</u>	(147) <u>A</u> <u>A</u> <u>I</u> <u>S</u> <u>A</u> <u>G</u> <u>D</u> <u>Q</u> <u>S</u> <u>I</u> <u>G</u> <u>D</u> <u>L</u> <u>I</u> <u>A</u> <u>E</u> <u>A</u> <u>M</u> <u>O</u> <u>K</u> <u>V</u> <u>G</u> <u>N</u> <u>E</u> <u>G</u> <u>V</u> <u>I</u> <u>T</u> <u>V</u> <u>E</u> (176) 66% :: : : ::: ::: ::: :::::	
65 KDa antigen of <u>M. leprae</u>	(147) <u>A</u> <u>A</u> <u>I</u> <u>S</u> <u>A</u> <u>G</u> <u>D</u> <u>Q</u> <u>S</u> <u>I</u> <u>G</u> <u>D</u> <u>L</u> <u>I</u> <u>A</u> <u>E</u> <u>A</u> <u>M</u> <u>O</u> <u>K</u> <u>V</u> <u>G</u> <u>N</u> <u>E</u> <u>G</u> <u>V</u> <u>I</u> <u>T</u> <u>V</u> <u>E</u> (176) 66% :: : : ::: ::: ::: :::::	
Mitochondrial protein P1 of chinese hamster and human	(175) <u>S</u> <u>A</u> <u>N</u> <u>G</u> -- <u>D</u> <u>K</u> <u>D</u> <u>I</u> <u>G</u> <u>N</u> <u>I</u> <u>I</u> <u>S</u> <u>D</u> <u>A</u> <u>M</u> <u>K</u> <u>K</u> <u>V</u> <u>G</u> <u>R</u> <u>K</u> <u>G</u> <u>V</u> <u>I</u> <u>T</u> <u>V</u> <u>K</u> (202) 64% : ::: ::: ::: ::: :::	
Rubisco subunit binding-protein alpha subunit of wheat	(151) <u>S</u> <u>A</u> <u>G</u> <u>N</u> -- <u>D</u> <u>E</u> <u>L</u> <u>I</u> <u>G</u> <u>A</u> <u>M</u> <u>I</u> <u>A</u> <u>D</u> <u>A</u> <u>I</u> <u>D</u> <u>K</u> <u>V</u> <u>G</u> <u>P</u> <u>O</u> <u>G</u> <u>V</u> <u>L</u> <u>S</u> <u>I</u> <u>E</u> (178) 57% : : : ::::: ::::: ::: :	
Putative 65 KDa chaperonin subunit of <u>Synechococcus</u> sp	(150) <u>S</u> <u>A</u> <u>G</u> <u>N</u> -- <u>D</u> <u>F</u> <u>E</u> <u>V</u> <u>G</u> <u>O</u> <u>M</u> <u>I</u> <u>A</u> <u>D</u> <u>A</u> <u>M</u> <u>O</u> <u>K</u> <u>V</u> <u>G</u> <u>K</u> <u>E</u> <u>G</u> <u>V</u> <u>I</u> <u>S</u> <u>L</u> <u>E</u> (177) 57%	

Figure 2. Regional alignment of PEB5 protein from C. jejuni strain 89-176 with heat shock proteins of other sources. Conserved residues between PEB5 and other heat proteins are indicated by ":". Residues conserved among all heat shock proteins are underlined. Number in parathesis represents position the sequence starts and ends at.

B. Publications:

Pei Z, Ellison RT III, Blaser MJ. Identification, purification and characterization of major antigenic proteins of *Campylobacter jejuni*. Journal of Biologic Chemistry 1991; 266:16363-16369.

Perlman DM, Jertborn M, Svennerholm AM, Blaser MJ. Serology of *Campylobacter enteritis* in Swedish musicians traveling to the Far East. In: GM Ruiz-Palacios, E Calva, BR Ruiz-Pallacios. *Campylobacter V: Proceedings of the Fifth International Workshop on Campylobacter Infections*. Instituto Nacional de la Nutricion, Mexico City 1991; 26-27.

Mascart-Lemonte F, Levy J, Blaser M, Oosterom J, Duchateau J, Hanson LA, Vaerman JP. Serum polymeric IgA (pIgA) antibodies are markers of acute intestinal bacterial infections. in Tsuchiya M, et al. eds. *Frontiers of Mucosal Immunology*. Elsevier Publishing, Amsterdam 1991; 269-270.

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Pei Z, Blaser MJ. Purification and characterization of a *Campylobacter coli* protein resembling a *C. jejuni* common antigen. Abstract presented at the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, September 1991.